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Promoter switching allows simultaneous transcription of LANA and K14/vGPCR of Kaposi's sarcoma-associated herpesvirus

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Abstract

Latent transcription of the latency-associated nuclear antigen (LANA/ORF73) of Kaposi's sarcoma-associated herpesvirus is driven by the LANAp-c. Complexity arises during lytic reactivation, however, as the bicistronic K14/vGPCR transcript initiates 32 bp downstream of LANAp-c in the opposite orientation. We identify an Rta/ORF50-inducible LANA promoter (LANAp-i) that is distinct from the LANAp-c. LANAp-c is unaffected by Rta/ORF50. Utilization of the second, downstream LANAp-i explains how LANA and K14/vGPCR are simultaneously transcribed during de novo infection or lytic reactivation. Transactivation of LANAp-i and K14/vGPCRp requires the C-terminal activation domain of Rta/ORF50 and is mediated by DNA-binding-dependent and -independent Rta/ORF50 mechanisms. Transcriptional profiling following viral reactivation support promoter reporter phenotypes. In sum, *cis*-elements within the LANAp were selected to ensure faithful expression of LANA and other genes regulated by LANAp during all stages of the KSHV lifecycle despite potential interference from K14/vGPCRp activity.

Keywords: KSHV; LANA; K14; vGPCR; Rta; ORF50; Transcription

Introduction

All Herpesviridae display a tightly regulated program of gene expression (reviewed in Roizman (1996)). KSHV is no exception and can enter one of two modes of infection: (a) latent infection in which the viral genome persists in the host cell with restricted viral gene expression and without cell destruction and (b) lytic infection in which all viral genes are expressed, infectious progeny are generated, and the host cell is destroyed. During latency, all KSHV-infected cells express the viral latency-associated nuclear antigen (LANA/ORF73) (Dittmer et al., 1998; Dupin et al., 1999). LANA is the predominant target of anti-KSHV antibodies in infected individuals. It is necessary and sufficient for latent viral episome persistence (Ballestas et al., 1999; Cotter and Robertson, 1999; Hu et al., 2002; Ye et al., 2004). Although LANA shows no homology at the DNA sequence level, its function and structural features are reminiscent of the Epstein Barr virus EBNA-1 and EBNA-2 proteins. LANA contains a central region of acidic repeats (EEDD, DE(E/Q)QQ, and LEEQEQEL between amino acids (aa) 338–840) a leucine zipper (LEEQEQEL at aa 840), a nuclear localization sequence and an N-terminal proline-rich region. The C-terminus of LANA binds to a 17-bp motif (LANA binding sites 1 and 2, LBS1 and LBS2) in the KSHV terminal repeats (TR) with a binding constant of 1.5nM. This motif is duplicated and repeated in the context of the TR. It is necessary and sufficient for latent episome replication (Garber et al., 2002, 2001). While LANA binds the KSHV genome through its C-terminus, LANA also binds to cellular chromatin and mitotic chromosomes. Binding to cellular chromatin and chromosomes is mediated through multiple mechanisms, primarily by LANA's N-terminus. In this manner, the LANA protein tethers the KSHV episome to cellular chromatin and chromosomes thereby ensuring proper segregation during host cell division (Ballestas et al., 1999; Cotter and Robertson, 1999; Kedes et al., 1997; Kellam et al., 1997; Moore and Chang, 1998; Rainbow et al., 1997; Szekely et al., 1999). Experimental abrogation of LANA expression through siRNA or genomic knockout leads to loss of KSHV episomes from latently infected cells (Godfrey et al., 2005; Ye et al., 2004).

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LANA transcription is regulated by the LANA promoter (LANAp) (Dittmer et al., 1998; Sarid et al., 1999; Talbot et al., 1999). Through alternative splicing, the LANA promoter also regulates expression of the viral cyclin D homolog (vCYC/ORF72) and a viral FLICE inhibitory protein (vFLIP/ORF71). With the exception of LANA-2/vIRF-3 in primary effusion lymphoma (PEL), all KSHV latency transcripts cluster to one major locus that includes LANA, vCYC, vFLIP, and Kaposin/K12 (Dittmer et al., 1998; Li et al., 2002; Sadler et al., 1999; Sarid et al., 1999; Talbot et al., 1999). This locus also encompasses the KSHV miRNAs (Cai et al., 2005; Pfeffer et al., 2005; Samols et al., 2005). Spatial clustering underscores the importance of this region and sets the latency-associated locus apart from all other viral transcription units. It is possible that during latency, all genes within the latency-associated locus can be derived by alternative splicing of nascent RNAs under control of the LANAp. Transcriptional profiling showed that during viral latency in PEL and in primary KS biopsies, LANA, vCYC, vFLIP, and Kaposin were constitutively expressed (Dittmer, 2003; Fakhari and Dittmer, 2002; Jenner et al., 2001; Paulose-Murphy et al., 2001). Under conditions where other KSHV promoters were silenced, the LANA promoter remained constitutively active. CpG islands within the LANAp are constitutively unmethylated in both PEL and KS (Chen et al., 2001) and are associated with an "open" chromatin environment. This is in contrast to the promoter for the viral immediate early protein Rta/ORF50. Treatment with sodium butyrate, an inhibitor of histone deacetylases (HDACs), did not change the acetylation status of histones H3 and H4 on the LANAp since the promoter was already de-repressed, contrasting the response observed on the Rta/ORF50 promoter and other regions of the viral genome (Lu et al., 2003). We previously reported a LANAp-*luciferase* reporter that showed constitutive activity in all cell lines tested including KSHV-positive BCBL-1 B-cells, KSHV-negative BJAB B-cells, HEK293 epithelial cells, and SLK endothelial cells (Jeong et al., 2001, 2004). Moreover, a 1861-bp DNA fragment originating at the LANA AUG at position 127,300 and extending to position 129,161 (−1299 bp relative to the latent transcription start site) was able to direct B-cell-specific reporter gene expression in transgenic mice (Jeong et al., 2002). This demonstrated that host cell transcription factors in the absence of any viral transactivators suffice to direct LANAp activity and, by inference, LANA, vCYC, vFLIP, Kaposin, and miRNA transcription during viral latency.

The LANAp is positively regulated by LANA itself through a consensus DNA-binding element within the minimal LANAp that is similar to the LBS1 within the KSHV TRs (Jeong et al., 2004). This establishes a self-stabilizing feedback loop to maintain KSHV latency (Wong et al., 2004; Renne et al., 2001; Chiou et al., 2002). In addition to specific DNA binding, LANA can act as a promiscuous transcription factor independent of its own DNA-binding recognition element through interaction with cellular proteins including: Sp-1 (Verma et al., 2004), RBP-jκ (also known as CSL) (Lan et al., 2005a), p53 (Friborg et al., 1999), Rb (Radkov et al., 2000), GSK-3β (Fujimuro et al., 2003), CBP (Lim et al., 2001), ATF4/CREB2 (Lim et al., 2000), as well as the chromatin modifying factors SAP30, mSin3A and CIR (Krithivas et al., 2000), meCP2, DEK (Krithivas et al., 2002), and Histone H1 (Cotter and Robertson, 1999).

Deletion analyses mapped the minimal core promoter region of the LANAp from −88 to +10 (nt 127,968–127,870) (Jeong et al., 2004) relative to the latent transcription start site at 127,880 (Dittmer et al., 1998). However, the presence of additional *cis*-elements flanking the core promoter significantly contributed to LANAp activity. Distal sequences from −88 up to −279 (nt 127,968–128,159) as well as sequences within the 5′-UTR from +10 down to +271 (nt 127,870–127,609) enhanced reporter activity more than 10-fold relative to the minimal core promoter (Jeong et al., 2004). Here, we explore the impact of these regions to LANAp activity during latency and in the presence of the KSHV lytic-switch protein Rta/ORF50. This report demonstrates the presence of a second, ORF-proximal LANAp (here termed the LANAp-i), which was first posited by Hayward and colleagues on the basis of sequence analysis (Nicholas et al., 1998) and more recently investigated by Matsumura et al. (2005). This new promoter was inactive during viral latency but, as opposed to the constitutive LANAp, was responsive to Rta/ORF50. Promoter switching between the constitutive LANAp and the novel LANAp-i provides a model how an overlapping lytic promoter region can act bidirectionally to initiate transcription of both the LANAp-i and the K14/vGPCR promoter (K14p), which directs transcription on the opposite strand in the opposite orientation. Here, we independently report and confirm the presence of the Rta/ORF50-inducible LANA promoter. We further show that the C-terminal domain of Rta/ORF50 is necessary for transactivation of the LANAp, and that an established Rta/ORF50 mutant with increased DNA-binding activity also enhanced activation of LANAp-i and K14p compared to wild-type Rta/ORF50. Yet, an Rta/ORF50 mutant that fails to bind DNA could still somewhat transactivate these promoters suggesting additional modes of regulation by Rta/ORF50 perhaps through cellular transcription factors, such as RBP-jκ.

Results

Basal LANA promoter activity

While the minimal core promoter region is defined from −88 to +10 bp, the inclusion of additional upstream or downstream sequences greatly increased reporter activity (Jeong et al., 2001, 2004). Of all prior deletion reporters of the LANAp, the +271/−279 luciferase reporter (*pDD83*, encompassing nucleotides 127,607–128,159) consistently gave the highest basal activity in all cell lines tested. Hence, we chose the reporter plasmid −279/+271 for further analysis. We have used plasmid nomenclature that describes the LANAp sequence that is present in the particular plasmid relative to the major latent LANA transcription start site at nt 127,880. Deletion mutants were derived by first generating novel *Nco*I, *Nhe*I or *Sac*I sites through targeted mutagenesis and then collapsing the reporter by restriction digest and subsequent re-ligation. LANAp deletion reporters that failed to yield significant activity were subcloned into empty reporter vector (pGL3basic) to exclude

any loss-of-function mutants in the luciferase open reading frame that may have unintentionally resulted from PCR. Fig. 1 shows a diagram of the parental LANAp plasmid +271/−279 and six downstream deletion mutants (panel A) and their constitutive reporter activity in HEK 293 cells (panel B). Deletion of +271 to +47 did not affect constitutive LANAp activity. However, deletion of 12 more bp up to +35 reduced LANAp activity to 20% that of the parental reporter +271/−279, a level that was still approximately 20-fold greater than background levels of the pGL3basic control. Further deletion of the LANA 5′-UTR up to and including the transcriptional start site at 127,880 completely abolished LANAp constitutive activity, as expected (mutant −1/−279). To verify these observations in an alternative cell line, we repeated these experiments in KS-tumor derived SLK cells. SLK cells are KSHV negative, of endothelial cell origin ($CD34^{pos}$, Ulex $Lectin^{pos}$) and form vessel-like structures in Matrigel (Herndier et al., 1994; Lunardi-Iskandar et al., 1995). These cells can be infected with KSHV and rapidly enter latency (Grundhoff and Ganem, 2004). By comparison to epithelial-derived, highly passaged HEK293 or HeLa cells, the rate of spurious transcription is much lower in SLK cells leading to minimal background reporter activity. The six 3′-LANAp deletion reporters produced a similar trend of constitutive promoter activity in SLK cells (Fig. 1C) compared to HEK293 cells (Fig 1B) with the exception of mutant +205/−279. Mutant +205/−279 displayed promoter activity comparable to parental reporter in HEK293 cells (Fig. 1B) but only approximately 23% activity compared to parental reporter in SLK cells, which still was greater than 100-fold of empty vector (Fig. 1C). In both cell lines, mutants +186/−279 and +141/−279 display lower promoter activity compared to parental reporter +271/−279. Further deletion with mutant +47/−279 resulted in a restoration of maximal promoter activity comparable to parental reporter +271/−279 in both cell lines. The reasons for these effects are currently unknown; however, these data implicate an active role for the 5′-UTR in regulation of latent LANAp activity.

The LANAp is active if regions −88 bp upstream of the 127,880 start site are present and inactive if only −57 bp upstream are present (Jeong et al., 2001). To further define the upstream region necessary for constitutive LANAp activity, a second series of LANAp deletion mutants with sequential deletion of upstream promoter region were assayed for basal activity. Fig. 2 shows a diagram of these upstream LANAp deletion mutants (panel A) and their reporter activity in HEK293 cells (panel B) and in SLK cells (panel C). We observed an increase in promoter activity of mutants +271/−238 and +271/−233 compared to the parental reporter +271/−279 in HEK293 cells (Fig. 2B). More important and consistent is the loss of ≥70% reporter activity if −160 bp or fewer are included. This defines −160 to −273 as an ancillary promoter regulatory region. Over 90% of reporter activity was lost if the TATA box was deleted (−50). The exception to this trend was a single deletion mutant +271/−93, which retained wild-type activity even though the adjacent deletion constructs (both 5′ and 3′) lost activity. Mutants +271/−65 and +271/−61 showed minimal promoter activity in both cells lines and further deletion from −50 all the way to +58 resulted in inactive promoters, thus further defining the 5′-boundary of the core LANAp and demonstrating that no other constitutive promoters are present within the +58 to +271 region.

LANA promoter response to Rta/ORF50

Having generated a panel of LANAp deletion mutants, some of which contain lytic K14/vGPCR promoter (K14p) elements, we investigated the effect of Rta/ORF50 co-expression on reporter activity in the context of a functional LANAp. The full-length LANAp consistently showed increased activity in the presence of Rta/ORF50 in 293 cells (Fig. 3B) and in SLK cells

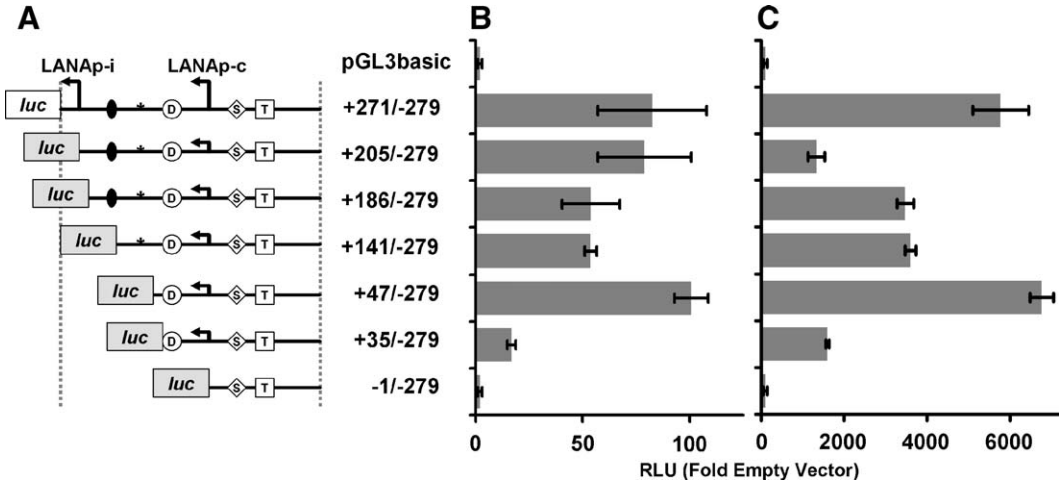

Fig. 1. Deletion analysis reveals that the constitutive 127,880 start site is required. (A) Schematic of LANAp reporters with sequential deletions spanning +205 up to −1. Indicated are the latent start-site (arrow, LANAp-c), Rta/ORF50-inducible start site at 127,610–11 (arrow, LANAp-i), RBP-jκ site (black oval), putative TATA element (asterisk), DPE (circle, D), Sp1 site (S, diamond), and LANAp-c TATA element (box). (B) Promoter activity of LANAp downstream deletion mutants in HEK293 cells. HEK293 cells were transfected with the indicated plasmids. Data represent the mean relative luciferase activity (RLU) after 48 h of triplicate experiments normalized by co-transfected β-galactosidase activity. SD, standard deviation is indicated by error bars. (C) Promoter activity of LANAp downstream deletion mutants in SLK cells. SLK cells were transfected with the indicated plasmids. Data are represented as in panel B.

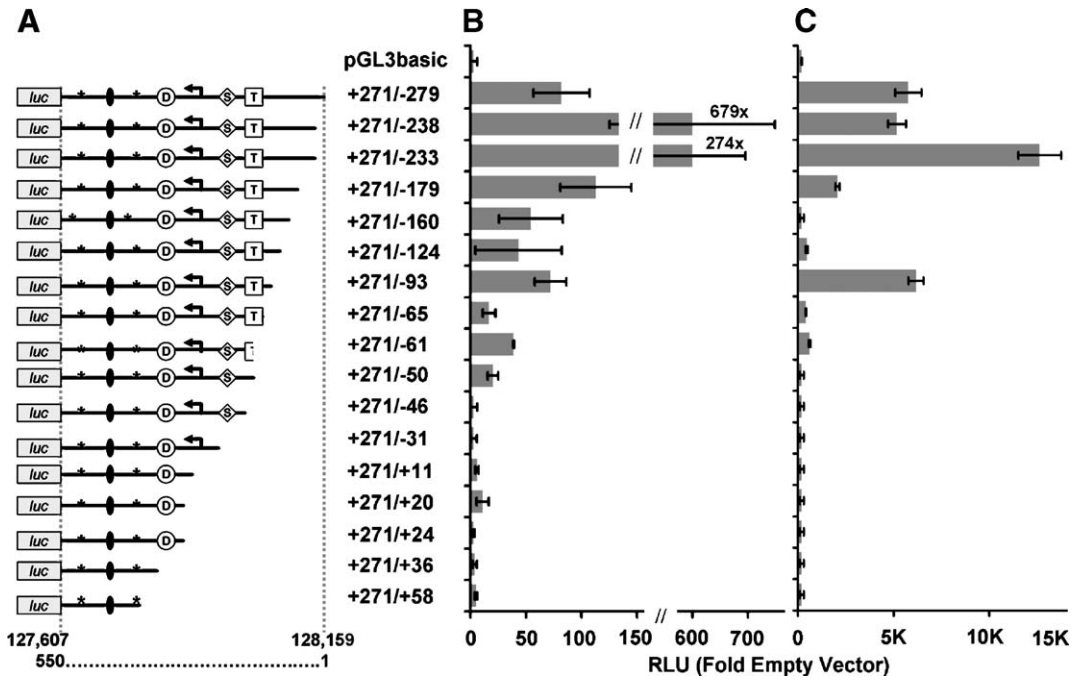

Fig. 2. The core LANAp is the only constitutive promoter within nt 127,607–128,159. (A) Schematic of LANAp reporters with sequential deletions spanning −279 down to +58. Indicated are the latent start-site (arrow), putative TATA elements (asterisk), RBP-jκ site (black oval), DPE (circle, D), Sp1 site (S, diamond), LANAp-c TATA element (box). (B) Promoter activity of in HEK293. Cells were transfected with the indicated plasmids. Data represent the mean relative luciferase activity (RLU) after 48 h of triplicate experiments normalized by co-transfected β-galactosidase activity. SD, standard deviation is indicated by error bars. (C) Promoter activity of in SLK cells. Cells were transfected with the indicated plasmids. Data represent the mean relative luciferase activity (RLU) after 48 h of triplicate experiments normalized by co-transfected β-galactosidase activity. SD, standard deviation is indicated by error bars.

(Fig. 3C). Activation by Rta/ORF50 was reproducible but low by comparison to bona-fide KSHV lytic promoters (this work and Damania et al., 2004). We first assayed our panel of downstream deletion mutants for Rta responsiveness in 293 cells and found that deletion of nt +271 to +205 decreased Rta/ORF50 responsiveness by 70–100%, demonstrating that this region (nt 127607–127675) was required for Rta responsiveness in 293 cells (Fig. 3B). In SLK cells, a similar trend was observed whereby deletion of the same region (nt +271 to +205) reduced Rta/ORF50 responsiveness by 70% (Fig. 3C); however, the overall response to Rta/ORF50 in SLK cells was greater than in 293 cells, therefore, despite a 70% decrease in activation by Rta/ORF50, a significant activation was still observed compared to basal activity of the +205/−279 reporter.

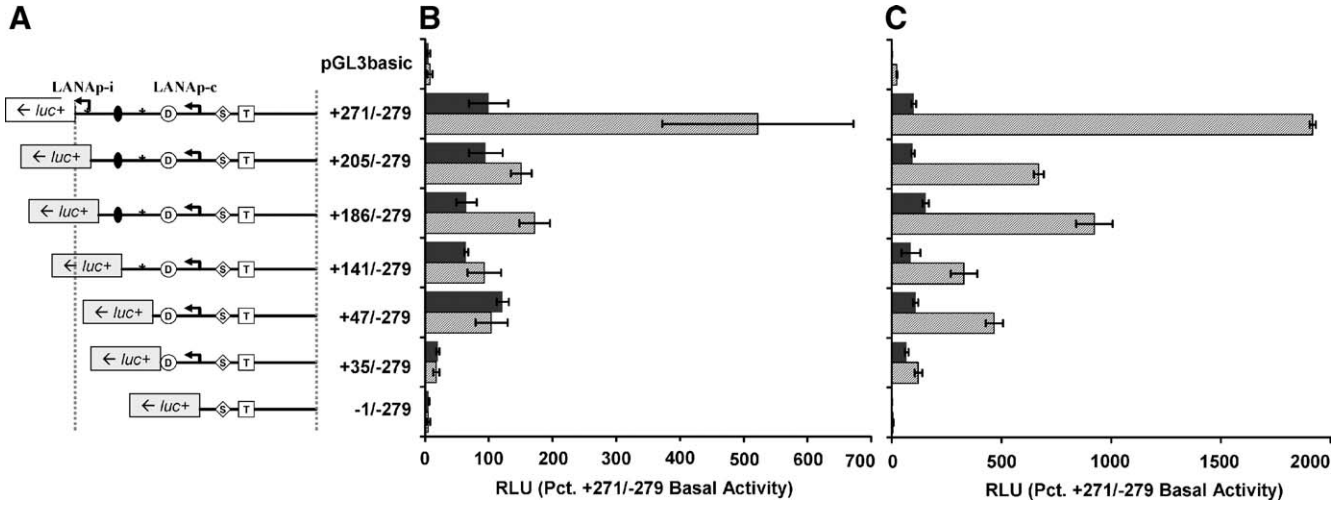

Fig. 3. Rta/ORF50 responsiveness of the LANAp requires nt 127,607–127,675. (A) Schematic of LANAp used in this study. Indicated are the latent start-site at 127,880 (arrow, LANAp-c) and lytic start site at 127,610–11 (arrow, LANAp-i), putative TATA elements (asterisks), RBP-jκ site (black oval), DPE (D, white oval), Sp1 site (S, diamond), and LANAp-c TATA element (T, box). (B and C) Promoter activity in the presence (hashed bars) or absence (black bars) of Rta/ORF50. HEK293 cells (panel B) or SLK cells (panel C) were transfected with the indicated reporter plasmids in the presence of either Rta/ORF50 or empty vector. Reporter activities were normalized to basal activity of parental +271/−279 LANAp activity; standard error is indicated by error bars.

Further deletion of the consensus RBP-jκ site (located at +139 to +145) decreased promoter activation by 80–100%. In both cell lines, the core latent LANAp was not activated by Rta/ORF50 in isolation (Figs. 3B and C, reporter +35/−279) demonstrating the Rta/ORF50-responsive elements are located downstream from the core latent LANA promoter.

We then assayed the upstream LANAp mutants in response to Rta/ORF50 expression in the same two cells lines (Figs. 4A and B); the relative position of these mutants is summarized in Fig. 4C. While mutants with less than −61 bp showed no constitutive promoter activity (Figs. 4A and B, mutants +271/−50 to +271/+58 black bars), upon expression of Rta/ORF50 the same reporters became active in both 293 and SLK cells (Figs. 4A and B, hashed bars). Indeed, five mutants that did not contain the minimal core LANAp (mutants +271/+11 to +271/+58) exhibited promoter activity in the presence of Rta/ORF50. The fold activation in response to Rta/ORF50 expression of these five Rta-dependent reporters was higher than for parental reporter +271/−279 (compare range of 8- to 22-fold activation for mutants +271/+11 to +271/+58 versus 5-fold for +271/−279, Fig. 4A, hashed bars). To distinguish between the latent constitutively active LANA promoter (mutant +47/−279) and the Rta/ORF50-dependent promoter region (mutant +271/+36), we will refer to them as the LANAp-c and LANAp-i (for constitutive and inducible), respectively.

In an effort to quantify the LANA promoter response to Rta/ORF50, we performed a dose-response experiment. We focused on the Rta-dependent LANAp-i or the latent LANAp-c individually or in their natural sequential context within the parental reporter construct +271/−279, which for simplicity from here on will be referred to as the LANAp-FL, for full-length (see Fig. 5A for diagram). The lytic K14 promoter reporter served as our positive control since it is Rta/ORF50-responsive (Chiou et al., 2002; Jeong et al., 2001; Liang and Ganem, 2004). As expected, the K14p was dose-responsive to increasing amounts of Rta/ORF50 protein (see Fig. 5B for luciferase activity and 5G for Rta/orf50 protein levels). Of note, within the K14p construct (which encompasses nt 127,297–

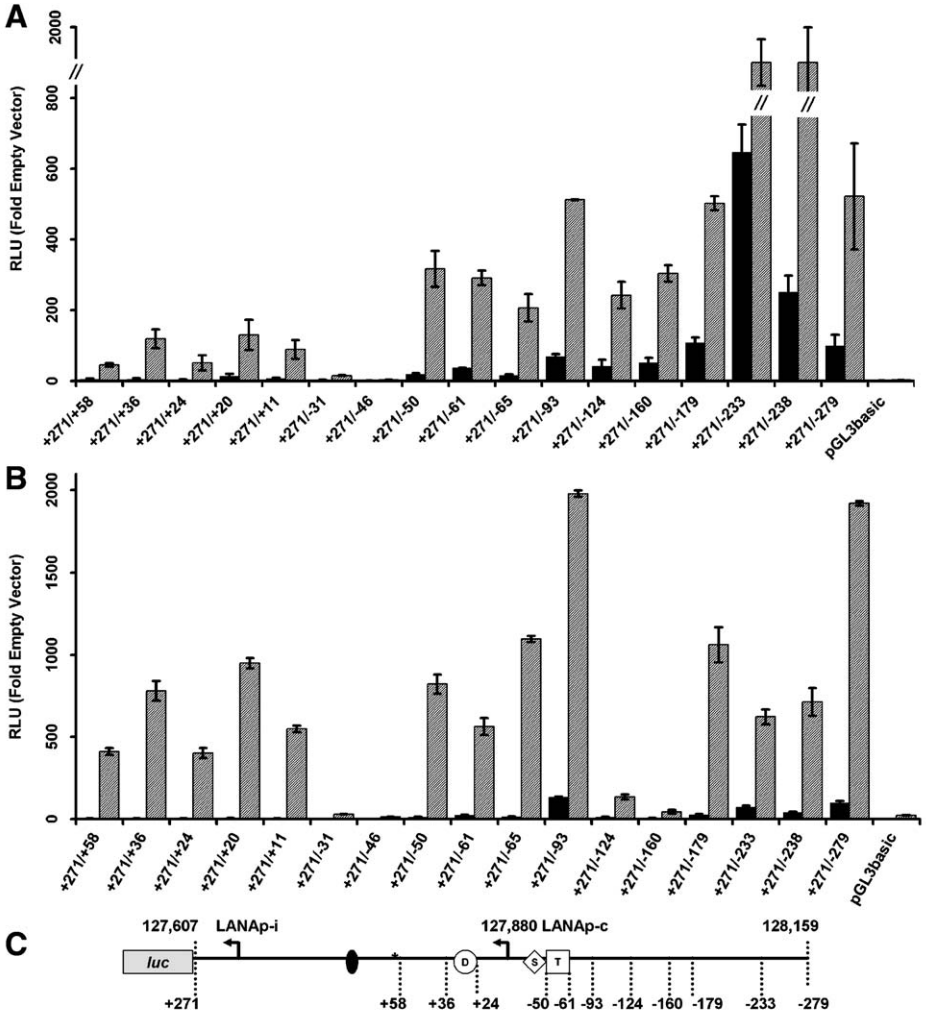

Fig. 4. Rta/ORF50 responsiveness of LANAp deletion mutants confirms the novel downstream LANA promoter, LANAp-i. (A and B) Promoter activity in HEK293 cells (panel A) and in SLK cells (panel B). Cells were transfected with the indicated reporter plasmids in the presence (hashed bars) or absence (black bars) of Rta/ORF50; standard error is indicated by error bars. (C) Schematic of LANAp for visual reference. Positions of representative deletions are shown below the dotted lines relative to the latent start site at nt 127,880.

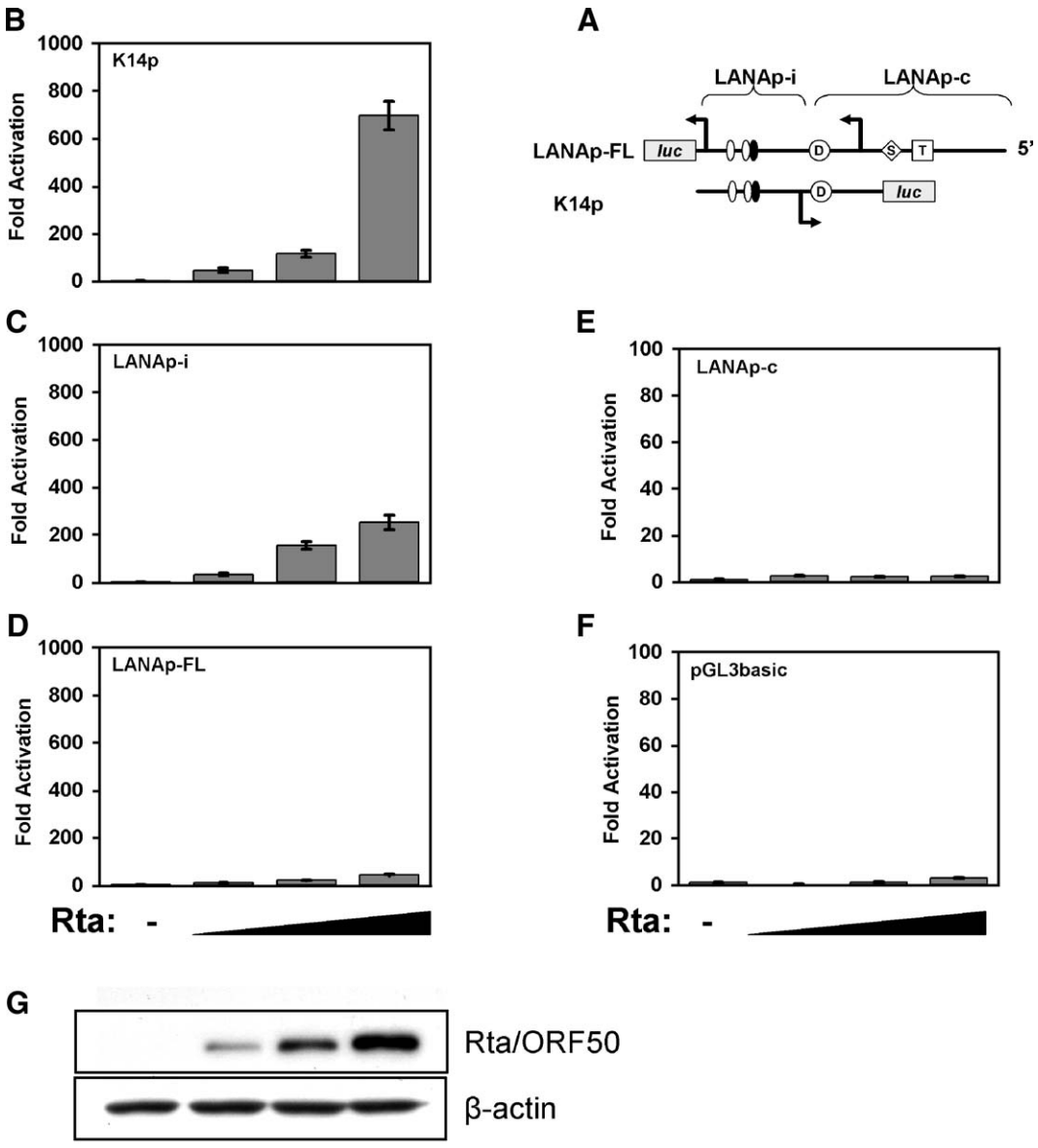

Fig. 5. The LANAp-i and K14p, but not LANAp-c are dose-responsive to Rta/ORF50. (A) Schematic of promoter reporters used in this assay. Transcription start sites are shown as arrows and indicate transcript orientation. RBP-jκ site (black oval), K14p Rta/ORF50-responsive elements (Chiou et al., 2002) (white ovals, black oval), DPE (D, white circle), Sp1 site (S, diamond), and LANAp-c TATA element (T, box) are depicted. (B) K14/vGPCR promoter activity shown as fold activation by Rta/ORF50 along the *y*-axis against increasing dose of Rta/ORF50 expression vector along the *x*-axis (0, 1:0.5, 1.1, 1:5 ratio of promoter reporter:expression vector); standard error is indicated by error bars. (C) LANAp-i promoter activity shown as described in panel B. (D) LANAp-FL promoter activity shown as described in panel B. (E) LANAp-c promoter activity shown as described in panel B; *note change in y-axis scale*. (F) Empty reporter (pGL3basic) background activity shown as described in panel B; *note change in y-axis scale*. (G) Detection of myc-tagged Rta/ORF50 and cellular beta-actin protein levels by immunoblot from a representative LANAp-i dose-response experiment.

127,886), the overlapping LANAp-c region is truncated at −6. Therefore, LANAp-c is inactive, and no interfering transcripts could be produced from the opposite strand. The K14p, LANAp-i, and LANAp-FL exhibited increased promoter activity with increasing amounts of Rta/ORF50 expression vector (Figs. 5B, C, and D). In contrast, no concentration of Rta/ORF50 expression vector had any effect on LANAp-c activity (Fig. 5E, note the change in scale). These data establish the presence of two separate promoters the LANAp-c and the LANAp-i that each respond differently to the lytic switch protein, Rta/ORF50. We also noticed the magnitude of Rta/ORF50-responsive promoters was quite different. The K14p was activated by Rta/ORF50 up to 695-fold by the same amount of Rta/ORF50 that activated the LANAp-i 251-fold and the LANAp-FL only 43-fold. This phenotype is consistent with the hypotheses (a) that sequences common to the LANAp-i and K14p (nt 127607–127845) mediate Rta/ORF50 responsiveness and (b) that the upstream constitutive LANAp-c (nt 127845–128159) inhibits Rta/ORF50 responsiveness of the LANAp-i in the context of the full-length promoter, perhaps due to promoter interference.

To visualize the latent and lytic phenotypes of each LANAp mutant, we plotted the basal constitutive promoter activity of

each LANAp deletion reporter against its fold activation by Rta/ORF50. As seen in Fig. 6, all of the "upstream" LANAp deletion reporters (circles) were responsive to Rta/ORF50 at least 5-fold (mutants +271/−238 to +271/+58), which is explained by the fact that each of these constructs contains the Rta-dependent LANAp-i region. However, only those "upstream" deletion reporters that also contained the latent LANAp-c start site at 127,880 displayed promoter activity in the absence of Rta/ORF50 (Fig. 6, mutants +271/−238 to +271/−31). In contrast, none of the "downstream" deletion mutants (squares) were activated by Rta/ORF50 though all retained basal promoter activity in the absence of Rta/ORF50 expression with the exception of one mutant, −1/−279, in which the latent LANAp-c start site at 127,880 was deleted. By summarizing the deletion mutant data in this manner, we observed the clear separation of phenotypes of the latent LANAp-c and the lytic LANAp-i. The overall number of different deletion mutants in these experiments exceeds all prior studies, and our quantitative analysis instills a high level of confidence in these observations.

We next assayed our panel of latent or lytic reporter plasmids in response to an Rta/ORF50 expression vector in which the C-terminal activation domain was deleted (Lukac et al., 1999). As previously reported by Lukac et al., the C-terminal 160 amino acids (aa 531–691) of wild-type Rta/ORF50 were deleted to generate the ORF50ΔSTAD construct, which results in expression of a truncated Rta/ORF50 protein that is incapable of transactivating Rta/ORF50-responsive promoters (Lukac et al., 1999). We found that the LANAp-FL, the LANAp-i, and the lytic K14p reporters were transactivated by wild-type Rta/ORF50 as expected (Fig. 7, panel B hashed bars), but the truncated Rta/ORF50 mutant ORF50ΔSTAD was unable to transactivate any of the reporters in this assay (Fig. 7, panel B, black bars). ORF50ΔSTAD expression did not reduce basal constitutive activity of the LANAp-FL. These data demonstrate that the C-terminal activation domain of Rta/ORF50 is necessary for the transactivation of the LANAp.

Having established that the C-terminal activation domain of Rta/ORF50 was necessary for transactivation of the LANAp-i, we wanted to further clarify the mechanism of transactivation by Rta/ORF50. Rta/ORF50 is known to transactivate promoters by non-mutually exclusive mechanisms (Chang and Miller, 2004; Chang et al., 2005; Song et al., 2003): either direct binding to DNA sequences or by protein–protein interactions with cellular transcription factors that mediate DNA binding. First, to test whether direct DNA binding is involved in LANAp transactivation by Rta/ORF50, we obtained established Rta/ORF50 DNA-binding mutants: the ORF50(KK/EE) mutant, which has increased affinity for binding Rta/ORF50 response element (RRE) DNA; the ORF50(R161A) mutant, which fails to bind RRE DNA; and the ORF50(KK/EE;R161A) mutant, which contains

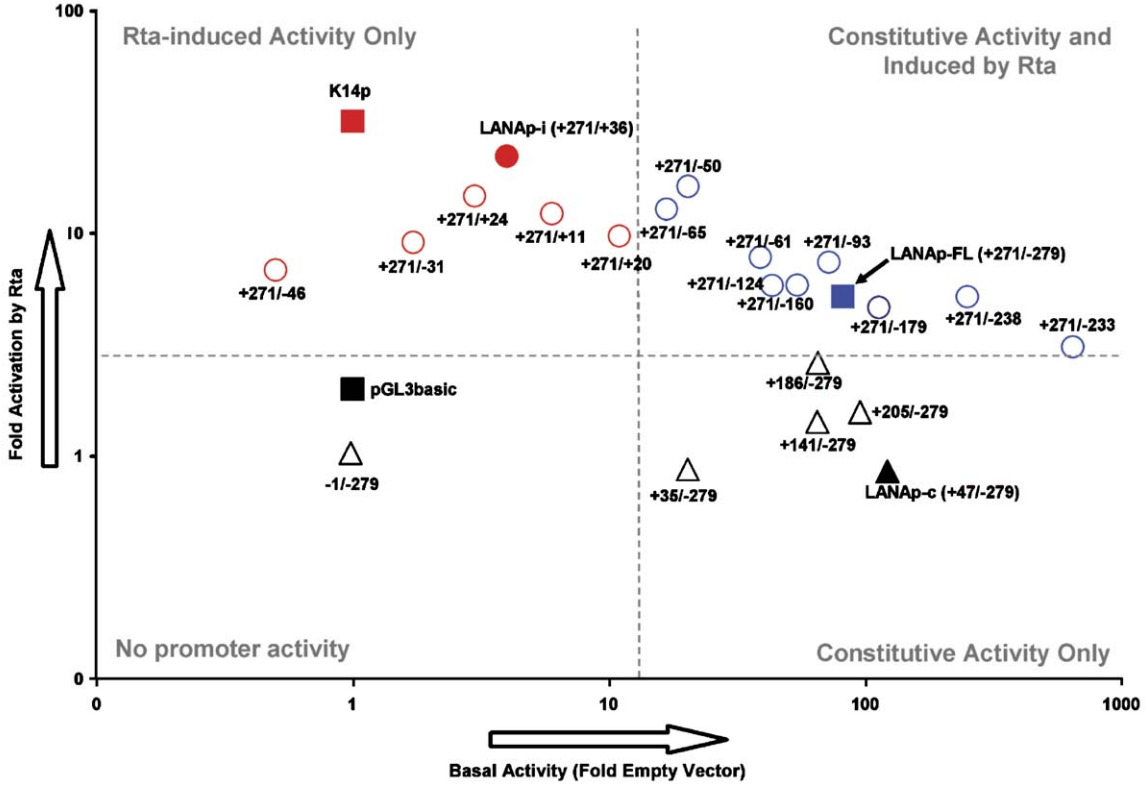

Fig. 6. Constitutive LANA promoter activity is distinct from Rta/ORF50 responsiveness. Summary LANAp reporter activity graphed as a measure of both fold activation by Rta/ORF50 along the *y*-coordinate and by basal activity along the *x*-coordinate. Reporters with deletions upstream of the latent start site are shown as circles; reporters with deletions downstream of the latent start site are shown as triangles. Previously characterized lytic (K14p) and latent (LANAp-FL) or empty vector (pGL3basic) are shown as filled squares for reference. (Of note, the LANAp-FL reporter contains both the LANAp-c and LANAp-i regions). Reporters with both basal and Rta/ORF50-induced activity are shown in blue, reporters with only Rta/ORF50-dependent activity are shown in red, and reporters that lack response to Rta/ORF50 expression are shown in black.

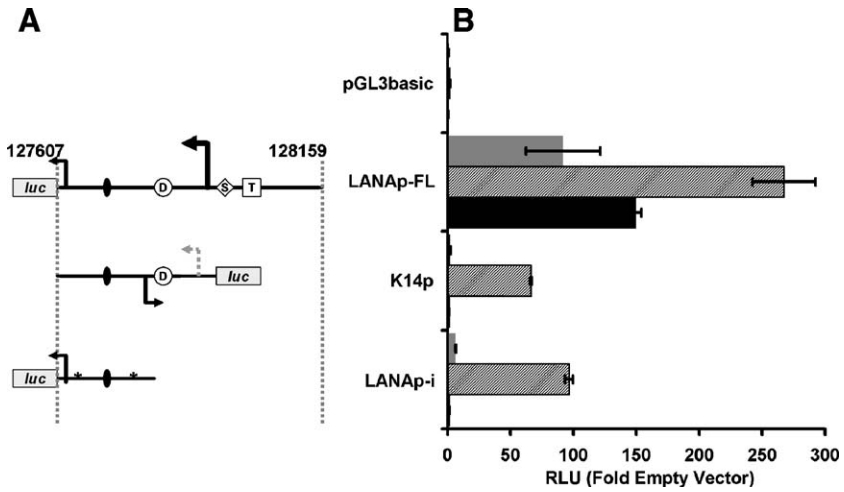

Fig. 7. Activation of LANAp-i and K14p requires the C-terminal activation domain of Rta/ORF50. (A) Schematic of promoter reporters used in this assay. Transcription start sites are shown as arrows and indicate transcript orientation. Putative TATA elements (asterisks), RBP-jκ site (black oval), DPE (D, white circle), Sp1 site (S, diamond), and LANAp-c TATA element (T, box) are depicted. (B) Promoter activity of Rta/ORF50-responsive promoters depicted as relative light units (RLU). HEK293 cells were transfected with the indicated reporter plasmids in the presence of Rta/ORF50 (hashed bars), ORF50ΔSTAD (black bars) or empty expression vector (gray bars); SE, standard error is indicated by error bars.

both mutations (Chang et al., 2005). We assayed the latent and lytic LANA promoters and the K14 promoter reporter in response to these Rta/ORF50 DNA-binding mutants. As before, the K14p and LANAp-i reporters were activated in response to wild type Rta/ORF50, the LANAp-c was not (Table 1). The LANAp-FL reporter, which contains both the LANAp-i and LANAp-c regions, displayed an intermediate phenotype compared to LANAp-i or LANAp-c alone. This result demonstrates that in the context of collinear orientation, the LANAp-c phenotype is dominant over the Rta/ORF50-responsive LANAp-i.

A similar response of the LANA-c and LANAp-FL was observed upon expression of the Rta/ORF50(KK/EE) enhanced DNA-binding mutant. However, a comparison of the two Rta/ORF50-dependent promoters, the LANAp-i and K14p, showed a dramatic increase in K14p response to the KK/EE mutant, which is not seen for the LANAp-i. This result indicates that while the LANAp-i and K14p share overlapping promoter elements and are both responsive the Rta/ORF50, the precise molecular mechanism of transactivation may in fact be different. Mutation in the conserved DNA-binding domain of Rta/ORF50 (R161A mutant) reduced fold activation of both the LANAp-i and K14 promoters but did not completely abolish transactivation. This result demonstrates a requirement for an intact DNA-binding domain of Rta/ORF50 in addition to the requirement for its C-terminal activation domain in LANAp-i and K14p transactivation.

Table 1
Fold activation in response to Rta/ORF50 DNA-binding mutants

Reporter	Fold activation		
	WT	KK/EE	R161A
LANAp-FL	10 ± 2	20 ± 4	7 ± 2
LANAp-i	126 ± 48	132 ± 8	36 ± 21
LANAp-c	2 ± 1	3 ± 1	2 ± 1
K14p	585 ± 95	2480 ± 247	147 ± 45

While mutation of the DNA-binding domain of Rta/ORF50 reduced activation of the Rta/ORF50-dependent LANAp-i and K14 promoters, it was not completely abolished which suggests that DNA-binding was not the only mechanism at work. Therefore we looked to *cis*-elements present within the overlapping LANAp-i and K14 promoters for cellular transcription factor binding sites that may contribute to Rta/ORF50 responsiveness. A consensus RBP-jκ site is located between the K14p and LANAp-i start sites and has been previously implicated in contributing to Rta/ORF50 responsiveness of these promoters (Lan et al., 2005a; Liang and Ganem, 2004; Matsumura et al., 2005) since Rta/ORF50 can bind to cellular RBP-jκ, which results in conversion of RBP-jκ from a transcriptional repressor to an activator.

We hypothesized that Rta/ORF50 may also modulate LANAp transcription by utilizing RBP-jκ sites located in the overlapping LANAp-i/K14p region at nt 127,736–127,740 (+140 to +144 with respect to the latent LANAp-c start site). To test this hypothesis, we generated LANAp-i and K14p reporter constructs in which this consensus RBP-jκ site was destroyed by site-directed mutagenesis. This mutation is different from the mutant reported by (mutant m4) Matsumura et al. (2005) in that while they substituted the wild-type LANAp sequence at 127,736–127,740 from 5′-ACCCT-3′ to 5′-TTAAG-3′ as we did, they in the process also created an additional deletion of nt 127,745, thus changing space relations as well as sequence. To maximize the assay range in our experiment, we used the Rta/ORF50(KK/EE) mutant that binds strongly to cognate DNA. Disruption of the RBP-jκ site in the both the LANAp-i and K14p reduced Rta/ORF50 responsiveness by 70 and 85%, respectively, compared to wild-type promoter (Table 2). A similar reduction (70% and 76%) in Rta/ORF50 responsiveness was observed following expression of the isogenic Rta/ORF50 mutant that contains the R161A mutation in its DNA-binding domain. Combining the *cis* RBP-jκ mutation in the LANAp-i and K14 promoters with the DNA-binding domain mutation of

Table 2
Fold activation of Rta-dependent promoters containing either wild-type (WT) or mutant (mut) RBP-jκ sites at nt 127736-127740 in response to Rta/ORF50 containing either wild-type or mutant (R161A) DNA-binding domain

Reporter	Fold activation		
	KK/EE	KK/EE(R161A)	Residual (%)
LANAp-i WT	23 ± 3	8 ± 2	30
LANAp-i mut	6 ± 1	4 ± 1	–
Residual	30%	–	20
K14p WT	406 ± 46	85 ± 1	24
K14p mut	56 ± 8	9 ± 1	–
Residual	15%	–	2

the *trans* factor Rta/ORF50 reduced fold activation of both promoters to greater levels compared to mutation of either the *cis* or *trans* element alone. This result demonstrates that in addition to the C-terminal activation domain and the DNA-binding domain of Rta/ORF50, the consensus RBP-jκ site within the overlapping LANAp-i and K14p contribute to Rta/ORF50 responsiveness.

Analysis of transcripts originating at LANAp-c or LANAp-i following KSHV reactivation

Because the LANAp-i start site (127610–11, Matsumura et al., 2005) is located within the first intron of the 5400 nt LANA (LT1) and 1700 nucleotide vCYC (LT2) spliced latent mRNAs originating at 127,880, we hypothesized that spliced latent messages are unaffected by Rta/ORF50 acting on LANAp-i. To test this hypothesis, we conducted array-based transcriptional profiling of all KSHV genes in KSHV-infected BCBL-1 primary effusion lymphoma (PEL) cells at early times (12 h post-induction) with three independent drugs known to reactivate KSHV: ionomycin, sodium butyrate, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Fig. 8). Therefore, only in the context of analyzing multiple viral mRNAs simultaneously we discerned mRNAs that change dramatically as a result of specific transactivation opposed to drug-induced non-specific effects. We designed a real-time QPCR primer pair that flanks the first LANA intron within the 5′-UTR and one that flanks the vCYC intron, thereby only producing a product if those introns are spliced out (Fakhari and Dittmer, 2002). The "vCYCspl" and "LANA 5′spl" spliced mRNAs were not significantly induced with any of the drug treatments (Fig. 8), confirming that the LANAp-c is not affected by lytic reactivation in the context of KSHV-infected B cells. The mRNA for Rta/ORF50 was induced following treatment with all three drugs, as expected. In contrast to the splice isoform-specific primers, primer pairs located within the vCYC open reading frame (ORF) and downstream of the LANA ORF ("LANA" primer, nt 123,626–123,688) detected spliced and unspliced LANA/vCYC/vFLIP mRNA, originating from LANAp-c or LANAp-i or any RNA species that use the splice vCYC splice acceptor at 123,776 and upstream splice donors, including RNA species that can give rise to the KSHV miRNAs (Cai et al., 2005; Pfeffer et al., 2005; Samols et al., 2005) by splicing from 123,594 to 118,779. Hence, this primer pair showed 5-fold induction comparable to LANAp-FL. This finding is consistent with our earlier observations using different time points that show among all the KSHV mRNAs the latency mRNAs originating at 127,880 change the least upon viral reactivation from PEL (Fakhari and Dittmer, 2002) and affirms the initial classification by Sarid et al. (1998).

Discussion

The LANA protein is essential for KSHV latent persistence. LANA and other genes, whose transcription is under control of LANAp, are thought to contribute to malignant transformation and survival of KS and PEL tumor cells (An et al., 2003; Chang

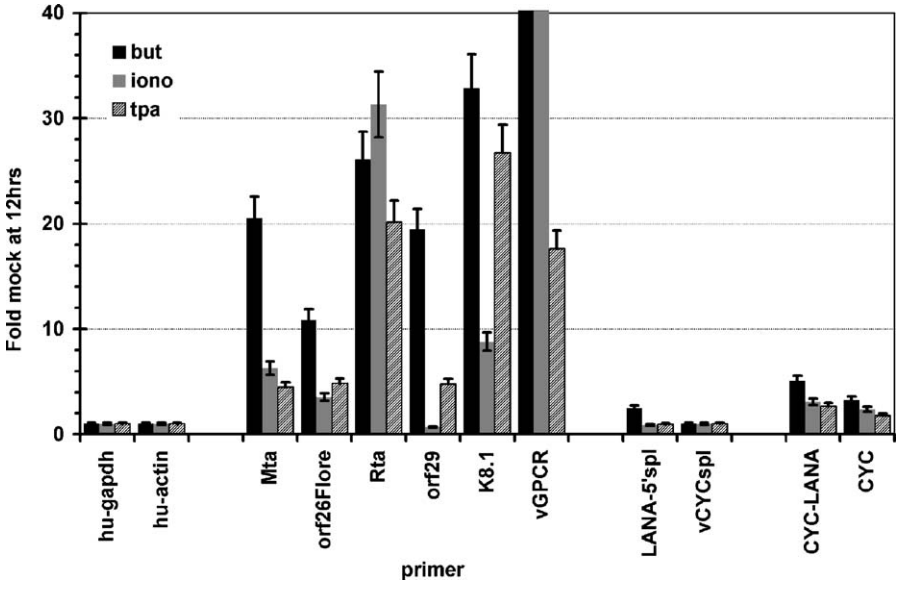

Fig. 8. The relative abundance of KSHV transcripts originating at either the latent LANAp-c or lytic LANAp-i following viral reactivation. Fold increase in mRNA levels in KSHV-infected BCBL-1 cells at 12 h post-treatment with either 20 ng/ml TPA (hashed bars), 500 nM ionomycin (gray bars), or 4 mM butyrate (black bars).

et al., 1996; Field et al., 2003; Godfrey et al., 2005; Guasparri et al., 2004). Hence, understanding the architecture and regulation of the LANAp will enhance our understanding of KSHV latency as well as yield insights into the development of LANAp-targeted anti-tumor and/or anti-KSHV drugs. Other than during asymptomatic latency in B cells (Dittmer et al., 1999; Mesri et al., 1996) or in KSHV-associated tumors (Dittmer et al., 1998; Dupin et al., 1999), LANA protein and LANA mRNAs have been detected immediately after de novo infection of endothelial cells and fibroblasts (Krishnan et al., 2004) (Yoo et al., 2005). In these, KSHV rapidly establishes latency but can be reactivated by TPA. LANA and Rta/ORF50 mRNAs were described as immediate early mRNAs. Within 24 h, Rta/ORF50 mRNA levels dropped below the level of detection, while LANA mRNA remained constant in the now 99% latently infected culture. This prompted the search for and eventual discovery of a novel lytic-phase LANA promoter (Lan et al., 2005a; Matsumura et al., 2005). We have confirmed this discovery herein.

We extend upon this observation by dissecting the mechanism of LANAp-i activation by Rta/ORF50 and the relation of LANAp-i to LANAp-c, as well as to the overlapping lytic K14 promoter. The LANAp-c is constitutively active during all forms of latency; its activity is enhanced by LANA (Jeong et al., 2004) and independent of Rta/ORF50 (Fig. 5). In contrast, the downstream LANAp-i is only active in the presence of Rta/ORF50 (Figs. 3-4). The major latent transcriptional start site at 127,880 and the DPE element at nt 127,850–127,856 (+30 to +24) are required for basal LANAp-c activity. Our sixteen "upstream" LANAp deletion mutants show that the LANAp-c region is dispensable for LANAp-i Rta/ORF50-dependent activity. Nucleotides 127,607–127,675 are sufficient and required for Rta/ORF50 responsiveness and encompass the core elements of the LANAp-i.

The LANAp-i was significantly more responsive to Rta/ORF50 in isolation than when linked to the LANAp-c as in the LANA-FL reporter (Figs. 3, 4 and 5). This effect could be a result of transcript elongation ensuing from the LANAp-c through the *LANA/ORF73* 5′-UTR (containing the LANAp-i), which might prevent initiation events on LANAp-i *cis* regions. Such a mechanism was previously reported for the *GAL10* and *GAL7* promoters of *Saccharomyces cerevisiae* and for transcription through tandem HIV-1 promoters (Greger et al., 1998; Greger and Proudfoot, 1998). In support of this notion, deletions of core LANAp-c regions that decreased basal promoter activity were associated with increased Rta/ORF50 responsiveness (Figs. 4 and 6). In contrast to our earlier finding (Jeong et al., 2004), we found no evidence for Rta/ORF50 directly repressing the LANAp-c at concentrations that are not globally toxic.

The LANAp-i and K14p, but not the LANAp-c, were activated by Rta/ORF50 expression in a dose-dependent manner (Fig. 5). The C-terminal activation domain of Rta/ORF50 was required for transactivation of the LANAp-i and K14p. The K14p was activated following expression of the enhanced consensus RRE DNA-binding Rta/ORF50(KK/EE) mutant, the LANAp-i was not. Expression of an isogenic mutant of Rta/ORF50 that abolishes RRE-mediated DNA binding reduced but did not completely abolish activation of the LANAp-i (Table 1). This is the first evidence that these two promoters that share an overlapping Rta/ORF50 responsive region may in fact be regulated by a different molecular mechanism, and that there may be directionality to Rta/ORF50 transactivation. Mutation of a consensus RBP-jκ site at 127,736–127,740 reduced the ability of Rta/ORF50 to transactivate both the LANAp-i and K14 promoters (Table 2), implicating that this site is required for the regulation of both promoters. These data suggest a mechanism whereby LANA transcripts derived from the LANAp-i can be transcribed during lytic reactivation without polymerase interference by K14/vGPCR transcripts that are simultaneously being transcribed on the complementary strand in the opposite orientation as a result of bidirectional transactivation from the KSHV lytic-switch protein, Rta/ORF50.

With respect to other KSHV latent transcripts, the discovery of the LANAp-i allowed us to test an interesting prediction. Because the LANAp-i is located within the 5′ intron of spliced transcripts originating from the LANAp-c (i.e., downstream of the splice donor site at nt 127,813) (Dittmer et al., 1998), mRNAs using this splice donor should not respond to Rta/ORF50 and should not change upon viral reactivation. This was indeed the case (Fig. 8). Transcripts initiating from the LANAp-i can only direct ORF73/LANA translation (as the most 5′ ORF) and ORF71/vFLIP translation through internal ribosomal binding (Bieleski and Talbot, 2001; Grundhoff and Ganem, 2001), as well as a potential mRNA encoding LANA and Kaposin through removal of the exon 123,594–118,779, which contains KSHV miRNAs (Cai et al., 2005; Pfeffer et al., 2005; Samols et al., 2005). This species would translate ORF73/LANA as the most 5′ ORF, but not Kaposin. This observation explains why Kaposin, which is induced and most likely required during lytic replication, has its own Rta/ORF50 responsive promoter and ORF-proximal transcription start site (Sadler et al., 1999). Alternatively, there may exist yet unexplored splice donor sites downstream from the LANAp-i that could regulate vCYC, Kaposin and the miRNAs that would not use the proximal splice acceptor just upstream of *LANA/ORF73* at either 127,477 (Dittmer et al., 1998) or 127,314 (Sarid et al., 1999).

During de novo infection, Rta/ORF50 is present within KSHV virions (Bechtel et al., 2005; Lan et al., 2005b) and as such is delivered into the host cell upon infection in the absence of LANA protein expression. Therefore, we speculate that Rta/ORF50 protein could initially transactivate the LANAp-i and K14/vGPCR promoters through direct DNA binding or via the shared consensus RBP-jκ site during de novo infection. As LANA protein expression ensues and LANA accumulates within the cell, expression of Rta/ORF50 protein is silenced as a result of LANA repression of the Rta/ORF50 promoter and LANA's inhibition of Rta/ORF50′s transactivation function (Lan et al., 2004). As a result, the LANAp-i and K14/vGPCR promoter activity would cease and LANA-coding mRNA could be transcribed from the latent LANAp-c, which is auto-regulated by LANA protein. This sequence of events can establish a positive feedback loop that is sufficient to initiate and maintain viral latency within a permissive cellular environment.

Materials and methods

Plasmids

All nucleotide sequence positions are based on the BC-1 KSHV isolate as sequenced by Russo et al. (1996). Plasmids +271/−279 (LANAp-FL, or pDD83 in previous reports), pDD104 (LANA expression vector), and pDD163 (vGPCR/K14p) were described previously (Jeong et al., 2001, 2002, 2004; Damania et al., 2004). Plasmid +271/−279 and derivatives were from a KS library (Zhong et al., 1996) and exhibit 100% sequence identity to the KSHV BC-1 sequence. Plasmid pDD163 was derived from BCBL-1 cells (Komanduri et al., 1996) and differs from the BC-1 sequence by a T to G substitution at nt 127,457. Deletion mutants were generated by first introducing 1-, 2-, or 3-bp substitutions to generate a novel *Nco*I, *Nhe*I or *Sac*I site (potential sites identified using PROLOG code (Dittmer, unpublished)) using the GeneTailor™ Site-Directed Mutagenesis System (Invitrogen, Carlsbad, CA) and the GeneAmp High Fidelity PCR System (Applied Biosystems, Foster City, CA). Positive substitution mutants were then subjected to restriction digest to delete a portion of the promoter sequence followed by subsequent re-ligation of the reporter construct. For site-directed mutagenesis, the +271/−279 LANAp-luciferase reporter served as the wild-type full-length template. To eliminate the possibility that inadvertent second-site mutations within the luciferase ORF might be responsible for loss of luciferase activity, the LANAp region of functionally inactive reporters was subcloned into empty pGL3 basic. The subcloned reporters displayed similar promoter activity compared to their respective parental reporter, demonstrating that loss of function was due to the intended mutation/deletion within the LANA promoter sequence (data not shown). All mutants were confirmed by DNA sequencing. The ORF50ΔSTAD expression vector was a kind gift from D. Lukac (Lukac et al., 1999); the wild-type ORF50 expression vector was a gift from B. Damania (Damania et al., 2004); the myc-tagged ORF50 expression vector was a gift from J. Jung; the ORF50(KK/EE), ORF50(R161A), and ORF50(KK/EE)(R161A) expression vectors were generous gifts from G. Miller (Chang et al., 2005).

Tissue culture and transfection

HEK293 cells were obtained from ATCC. SLK cells were previously described (Herndier et al., 1994). All adherent cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (0.05 μg/ml), and streptomycin (5 U/ml) (Invitrogen Inc., Carlsbad, CA) at 37 °C under 5% CO_2. At day 1, cells were seeded 2–5 × 10^4 cells/ml/well (12 well plate) to reach 50% confluency after 24 h. At day 2, 4000 ng of total DNA was suspended in 150 μl of DMEM (no serum, no antibiotics) and 10 μl of Superfect (Qiagen Inc., Valencia, CA). The transfection mixture was incubated for 30 min at room temperature, the total volume adjusted to 1.5 ml with complete medium and 0.5 ml of transfection mixture was added per triplicate well. Cells were incubated overnight at 37 °C under 5% CO_2, the medium was exchanged with fresh complete medium and then harvested 48 h post-transfection.

Immunoblotting

293 cells were first seeded in 10-cm plates then harvested 24 h post-infection in Cell Culture Lysis Reagent (Promega, Madison, WI); protein concentration of lysates were normalized by BCA assay (Pierce, Rockford, IL), and the lysate was subjected to luciferase assay (described below) and separation by 8% SDS-PAGE, transferred to PVDF membrane then blocked in 5% non-fat milk in PBST (0.05% Tween-20). Antibodies were diluted in blocking solution at 1:5000 for anti-myc-HRP (Invitrogen Inc., Carlsbad, CA), 1:10,000 for monoclonal anti-beta-actin (clone AC-15) (Sigma, St. Louis, MS), and 1:15,000 for goat anti-mouse-HRP (Santa Cruz Biotechnology, Santa Cruz, CA).

Luciferase activity

Firefly luciferase activity was measured 48 h post-transfection using the Luciferase Assay System (Promega, Madison, WI) in a BMG Labtech FLUOstar OPTIMA 96-well luminometer with automatic injector according to the manufacturers' instructions. β-Galactosidase activity was determined using Galacto-Light Plus Kit (Tropix, Bedford, MA) according to manufacturers' recommendations. Promoter reporter activities were normalized to β-galactosidase activity in Figs. 1 and 2. However, we observed that expression of Rta/ORF50 consistently and significantly affected β-galactosidase expression, skewing this value as a normalizing factor (data not shown). Therefore, for experiments where Rta/ORF50 was expressed, promoter activities were normalized to LANAp-FL (pDDD83) or pGL3 basic activity. Each series of mutants was transfected within 3 separate days, each time in triplicate wells. Each well was lysed and analyzed for luciferase and β-galactosidase expression independently. All luciferase counts were within the linear range of the assay, were measured at 0.5-s intervals over 10 s, and final values were derived as the average of interval readings after the luciferase signal had reached a steady plateau (2–10 s post-substrate addition).

Real-time quantitative PCR

Quantitative real-time PCR primers were designed using Primer Express 1.5 (Applied Biosystems, Foster City, CA) and used as described previously (Dittmer, 2003; Fakhari and Dittmer, 2002) on an ABI PRIZM 5700 Sequence Detector (Applied Biosystems, Foster City, CA) using universal cycling conditions (2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C). The cycle threshold values were determined by automated analysis. The threshold was set to five times the SD of the non-template control. Dissociation curves were recorded after each run, and the amplified products were routinely analyzed by 2% agarose gel electrophoresis (data not shown).

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